BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07K 16/20, G01N 33/569, 33/577

A1

(11) International Publication Number: WO 97/08204

(43) International Publication Date: 6 March 1997 (06.03.97)

(21) International Application Number: PCT/AU96/00543

(22) International Filing Date: 30 August 1996 (30.08.96)

(30) Priority Data:
PN 5146 30 August 1995 (30.08.95) AU

(71) Applicants *(for all designated States except US)*: MACQUARIE RESEARCH LTD. [AU/AU]; Macquarie University, NSW 2109 (AU). AUSTRALIAN WATER TECHNOLOGIES PTY LTD [AU/AU]; Level 1, 339-341 Pitt Street, Sydney, NSW 2000 (AU).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: VESEY, Graham [GB/AU]; 10 Plunkett Street, Drummoyne, NSW 2047 (AU). WILLIAMS, Keith [AU/AU]; 23 Nandi Avenue, Frenchs Forest, NSW 2086 (AU). VEAL, Duncan [GB/AU]; 70 Finlay Road, Turramurra, NSW 2074 (AU). CHAMPION, Alan [AU/AU]; 2 Fairview Street, Woy Woy, NSW 2256 (AU). PERERVA, Natalia [AU/AU]; 18 Beaumont Crescent, Bayview, NSW 2104 (AU).

(74) Agent: F.B. RICE & CO.; 28A Montague Street, Balmain, NSW 2041 (AU).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: METHODS FOR DETECTION OF CRYPTOSPORIDIUM OOCYSTS

(57) Abstract

A method of detecting the presence of viable *Cryptosporidium* oocysts in a sample containing *Cryptosporidium* oocysts, the method comprising the steps of a) treating the sample so as to cause any viable oocysts of *Cryptosporidium* in the sample to excyst, b) exposing the treated sample to an antibody that binds specifically to recently excysted *Cryptosporidium* oocysts such that the antibody binds to recently excysted *Cryptosporidium* oocysts in the sample, and c) detecting the presence of oocyst-bound antibody in the sample.

Methods for Detection of *Cryptosporidium* Oocysts

Technical Field

The present invention relates to antibodies reactive to recently excysted oocysts of *Cryptosporidium* and methods of detecting viable *Cryptosporidium* oocysts using the antibodies.

Background Art

The protozoan parasite *Cryptosporidium* is amongst the most common pathogens responsible for diarrhoeal disease in humans (Current 1986). Infection occurs when *Cryptosporidium* oocysts shed in the faeces of infected individuals are ingested by new hosts. Recently, several large outbreaks of cryptosporidiosis have occurred in which drinking water has been identified as the source of infection (Smith and Rose 1990, Badenoch 1990). Surveys have shown that many surface water supplies are contaminated with *Cryptosporidium* oocysts (LeChevallier *et al* 1991, Rose 1988).

The detection of *Cryptosporidium* oocysts in water relies on the concentration of particulate matter including oocysts from large volumes of water prior to staining with fluorescently labelled antibodies. Until recently, detection and identification of fluorescently labelled oocysts required examination of the sample using epifluorescence microscopy. The tedious and labour intensive nature of this detection method, in particular the amount of fluorescent microscopy required, limited the monitoring work which could be performed. The development of flow cytometric detection methods has alleviated many of these problems and enabled the routine monitoring of water for the presence of *Cryptosporidium* oocysts (Vesey *et al* 1994). A major limitation of this methodology, however, is the lack of oocyst viability measurements.

A further problem with the currently employed method for flow cytometric analysis of water samples for *Cryptosporidium* is the requirement of a flow cytometer that can sort particles for subsequent microscopical examination. Sorting flow cytometers are expensive and sophisticated instruments that require a highly skilled operator.

The flow cytometric method involves staining of samples with a fluorescently labelled monoclonal antibody specific to the surface of *Cryptosporidium* oocysts and then analysis with a sorter flow cytometer. Particles with the fluorescence and light scatter characteristics of *Cryptosporidium* oocysts are sorted onto a microscope slide and examined

b) exposing the treated sample to an antibody that binds specifically to recently excysted *Cryptosporidium* oocysts such that the antibody binds to recently excysted *Cryptosporidium* oocysts in the sample; and

c) detecting the presence of oocyst-bound antibody in the sample.

It will be appreciated that any treatment of the sample that causes oocysts to undergo excystation would be suitable. It is, however, presently preferred that the oocysts are caused to excyst by incubating the sample at about 37°C under acidic conditions, followed by incubating the sample under neutral to alkaline conditions at about 37°C. More preferably, the oocysts are caused to excyst by incubating the sample at 37°C at pH 2 to 4 for 10 to 60 minutes, followed by incubating the sample at 37°C at pH 7 to 9 for 10 to 60 minutes. The sample can be washed between the steps to facilitate the removal of the buffers and replacement with fresh buffers at the required pH.

Recently excysted *Cryptosporidium* oocysts are defined as oocysts that have excysted within several hours from treatment. Usually, the oocysts are exposed to antibody within one hour or less from being excysted to ensure optimal binding. It has been found that the antigen or antigens present on recently excysted oocysts to which antibodies can be made do not remain intact over prolonged periods. It will be appreciated that this period can be increased if the treated sample or oocysts are preserved in some manner. For example, freezing of the sample has been found to preserve the antigenicity of the excysted oocyst past this several hour period.

The present inventors have found that short-lived antigens are present on recently excysted *Cryptosporidium* oocysts and that specific antibodies can be raised against these antigens. These antibodies can be used to detect viable *Cryptosporidium* oocysts in samples. It will be appreciated that by following the teaching of the present invention useful antibodies can be produced against recently excysted *Cryptosporidium* oocysts.

In a preferred embodiment of the first aspect of the present invention, the sample is analysed by flow cytometry or microscopy to detect the oocyst-bound antibody. The binding of the antibody to the recently excysted oocysts can be measured indirectly by further treating the sample with a fluorescently-labelled ligand that binds specifically to the antibody and measuring the binding of the labelled ligand to the oocyte-bound antibody. Alternatively, the antibody can be fluorescently labelled prior to use and the binding of the

Brief Description of the Drawings

Figure 1 shows flow cytometric analysis of pure excysted oocysts labelled with monoclonal antibodies;

Figure 2 shows flow cytometric analysis of pure non-excysted oocysts reacted with monoclonal antibodies;

Figure 3 shows flow cytometric analysis of pure excysted oocysts stained with Cry4; and

Figure 4 shows flow cytometric analysis of environmental samples seeded with excysted oocysts and treated with two antibodies Cry26 and Cry4;

Modes for Carrying Out the Invention

MATERIALS AND METHODS

***Cryptosporidium* oocysts.** *Cryptosporidium parvum* oocysts cultured in lambs and purified by density gradient centrifugation were purchased from the Moredun Animal Research Institute, Edinburgh.

Monoclonal antibodies. Two female balbC mice were injected with oocyst preparations as presented in Table 1.. Mice were sacrificed, spleen cells dissected and fused with NS1 mouse myeloma cells and the resulting hybridomas cloned. Clones were screened for anti-oocyst antibody production by screening against fresh and excysted oocyst preparations with flow cytometry.

Excysted oocysts were prepared by excysting oocysts and then washing in saline solution.

Excysted SDS treated oocysts were prepared by excysting oocysts, treating with 1% (w/v) sodium deoxycholate at 21°C for 10 minutes and then washing in saline solution.

with non-excysted oocysts. This demonstrates that the antigens, recognised by these antibodies, are only accessible in open oocysts and are therefore internal.

Oocysts 10^5 (existed or fresh) were aliquotted into each well of a 96 well plate, and 100 µl hybridoma super supernatant added plus 10 µl of FITC-coupled sheep anti-mouse antibody (1/40 dilution) Silenus. Samples were incubated at 37°C for 30 minutes, then mixed with 200 µl of phosphate buffered saline and analysed by flow cytometry (Vesey, et al, 1994B). The flow cytometer was calibrated with dilutions of a commercially available anti-*Cryptosporidium* antibody so that positive and negative controls were defined.

Analysis of all 41 clones by ELISA, indirect immuno-fluorescence and Western blot produced the results presented in Table 2. All antibodies were positive by ELISA, with results ranging from weakly positive (+) to strongly positive (++++). A range of antigen sites were identified by immunofluorescence including sporozoites, oocyst walls and the interior of oocysts. A series of different antigen-binding patterns were identified by Western blot analysis.

Western blots of *Cryptosporidium parvum* antigens were probed with hybridoma supernatants containing monoclonal antibodies. Solubilised intact oocyst proteins were separated by SDS-polyaccrylamide gel electrophoresis under reducing conditions. Each lane consisted of 1×10^6 oocysts and detection of bound antibodies was by HRP-conjugated anti-mouse antibodies and 4-Chloro-1-napthol. A series of different antigen-binding patterns were identified by Western blot analysis. Each group of antibodies that reacted to a particular antigen on a given site on the oocyst (for example interior, surface, wall, sporozoite) had a characteristc protein binding pattern.

Antibodies Cry26 and Cry4 were purified using EZ-Sep (Amrad Phamacia Biotech, Boronia. Australia) according to the manufacturer's instructions. Purified Cry26 antibody was conjugated with CY3 (Biological Detection Systems, PA, USA) according to the manufacturer's instructions.

Oocyst preparation. Oocysts (1×10^8) were surfaced sterilised by suspending in 1 ml of 70% (v/v) ethanol for 5 min and then washing by centrifuging at 13000g for 2 min, discarding the supernatant and resuspending in phosphate buffered saline (PBS), pH 7.4. Excystation was then performed by suspending in 1 ml of acidified PBS, pH 2.75, incubating for 30 min at 37°C, then washing by centrifuging at 13000g for 2 min and resuspending in PBS, pH 7.4 with 0.1% (w/v) sodium deoxycholate and 0.22% sodium hydrogen carbonate. After a further 30 min incubation at 37°C the sample was centrifuged at 13000g for 5 min and fixed by resuspension in 1 ml of PBS, pH 7.4 with 1% (v/v) formalin (excysted oocyst suspension).

Staining pure oocysts. An aliquot (10 µl -10^6 oocysts) of excysted oocyst suspension was mixed with 200 µl of Cry4 (approximately 0.005 mg/ml) and incubated at 37°C for 10 min prior to the addition of 5 µl of a goat anti-mouse FITC conjugated antibody (Silenus Laboratory, Melbourne, Australia; Product DDAF). After a further 10 min incubation at 37°C the sample was analysed by flow cytometry.

Staining seeded environmental samples. An aliquot (10 µl) of excysted oocyst suspension that had been stained with Cry4 as above was mixed with 500 µl (equivalent to 5 litres unconcentrated sample) of a river water sample that had been concentrated by flocculation (Vesey *et al* 1994A) and fixed in 4% (v/v) formalin.

Filtered (0.22 µm) bovine serum albumin fraction V was then added to a final concentration of 2% (w/v) before the addition of 20 µl of CY3 conjugated Cry26 antibody (approximately 0.055 mg/ml). The sample was incubated at 37° for 10 min and then analysed by flow cytometry.

Flow cytometry analysis. A coulter Elite flow cytometer (Coulter Corporation, Miami, USA) and Becton Dickinson Facscan flow cytometer (Becton Dickinson, San Francisco, USA) were used to analyse samples as described previously (Vesey *et al* 1994B). Sorted samples were further examined using epifluorescence microscopy.

numbers of motile bacteria were observed in these samples, whereas no motile bacteria were observed in samples that had been treated with ethanol.

Results of the analysis of environmental water samples that had been seeded with oocysts stained with both the Cry26 and Cry4 antibodies are presented in Figure 4. The first graph represents side scatter versus red fluorescence (ie, the fluorescence due to binding of CY3 labelled Cry26 and the second graph represents side scatter versus green fluorescence (ie, the fluorescence due to binding of FITC labelled Cry4). A box was drawn on the first graph around an area containing the stained oocysts. This box was then used to gate graph 2 (ie, the only particles that appear on graph 2 are those that appeared in the box). Two distinct populations are present on graph 2, highly fluorescent viable oocysts and non-fluorescent, non-viable oocysts and debris. The viable oocysts are completely separated from all other particles, thus allowing enumeration.

Cryptosporidium oocysts are surrounded by an extremely robust oocyst wall that can protect the organism in the environment. When a viable oocyst is exposed to a temperature of 37°C in acidic solution, followed by an alkaline solution, the sporozoites rapidly break out of the oocysts wall and swim away leaving behind an empty oocyst (Current 1986).

The monoclonal antibody Cry4 recognises an internal antigen in empty *Cryptosporidium* oocysts. The antigen is not accessible in oocysts that have not excysted nor is it present in oocysts that have excysted prior to the excystation treatment. Furthermore, the antigen recognised by Cry4 is removed if oocysts are excysted in the presence of bacteria. This would indicate that the antigen is destroyed by bacterial enzymes.

Previously, oocyst viability has been determined on pure samples of oocysts by performing excystation and then manually counting the number of full and empty oocysts. This method is tedious and labour intensive. The development of the antibody Cry4 or other antibodies that react specifically with recently excysted oocysts will enable immunofluorescence assays employing flow cytometry or other automated technologies to replace this manual methodology.

Methods for determining the viability of small numbers of *Cryptosporidium* oocysts based on the uptake or exclusion of the fluorogenic vital dyes propridium iodide (PI) and 4'6-diamidino-2-phenylindole (DAPI) have been reported (Campbell *et al* 1992). The authors report that dead

the oocyst wall (Moore *et al* 1995). By performing excystation and then staining with an antibody which reacts specifically to recently excysted oocysts and a surface antibody according to on method of the present invention it is now possible to dual label oocysts and detect them using a simple analysis-only flow cytometer.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Claims

1. A method of detecting the presence of viable *Cryptosporidium* oocysts in a sample containing *Cryptosporidium* oocysts, the method comprising the steps of:

a) treating the sample so as to cause any viable oocysts of *Cryptosporidium* in the sample to excyst;

b) exposing the treated sample to an antibody that binds specifically to recently excysted *Cryptosporidium* oocysts such that the antibody binds to recently excysted *Cryptosporidium* oocysts in the sample; and

c) detecting the presence of oocyst-bound antibody in the sample.

2. The method according to claim 1 such that the *Cryptosporidium* is *Cryptosporidium parvum*.

3. The method according to claim 1 or 2 such that the oocysts are caused to excyst by incubating the sample at about 37°C under acidic conditions, followed by incubating the sample under neutral to alkaline conditions at about 37°C.

4. The method of claim 3 wherein the oocysts are caused to excyst by incubating the sample at 37°C at pH 2 to 4 for 10 to 60 minutes, followed by incubating the sample at 37°C at pH 7 to 9 for 10 to 60 minutes.

5. The method of claim 4 wherein the oocysts are caused to excyst by incubating the sample at 37°C at pH 2.75 for 30 minutes, followed by incubating the sample at 37°C at pH 7.4 for 30 minutes.

6. The method according to any one of claims 1 to 5 wherein the recently excysted *Cryptosporidium* oocysts are excysted oocysts in the sample up to about one hour after the treatment of step (a).

7. The method according to any one of claims 1 to 6 such that the antibody is fluorescently labelled prior to use and the binding of the antibody to the recently excysted oocysts is detected by measuring directly the fluorescence of the oocyte-bound antibody.

8. The method according to any one of claims 1 to 7 such that the binding of the antibody to the recently excysted oocysts is measured indirectly by further treating the sample with a fluorescently-labelled ligand that binds specifically to the antibody and measuring the binding of the labelled ligand to the oocyte-bound antibody.

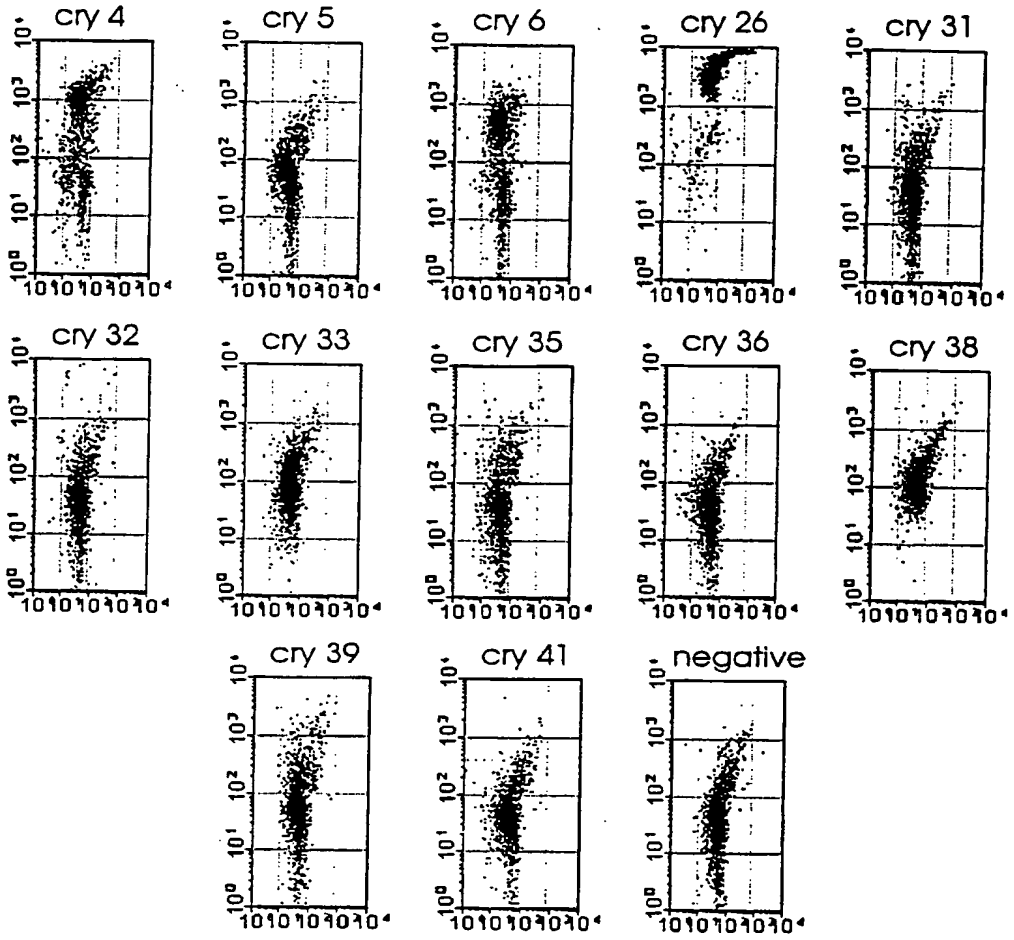
cry 4
cry 5
cry 6
cry 26
cry 31
cry 32
cry 33
cry 35
cry 36
cry 38
cry 39
cry 41
negative

Fig. 1

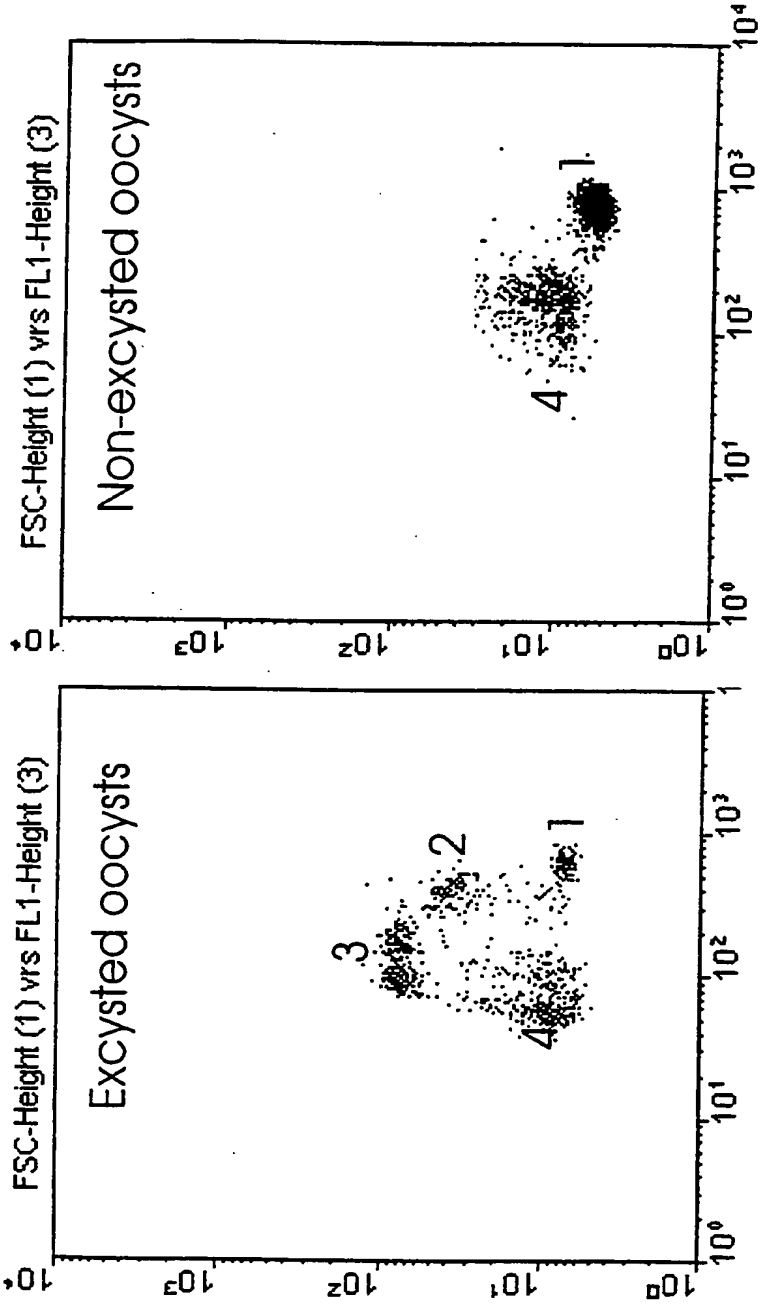
Non-excysted oocysts
FSC-Height (1) vrs FL1-Height (3)
1
4
Excysted oocysts
FSC-Height (1) vrs FL1-Height (3)
1
2
3
4

Fig. 3

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU96/00543

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C07K 16/20 G01N 33/569 33/577

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
WPAT, CHEMICAL ABSTRACTS (See Keywords in the electronic database box below)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
JAPIO, USPM, MEDLINE (See Keywords in the electronic database box below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT, JAPIO, USPM, KEYWORDS: CRYPTOSPORIDI: and OOCYST#
MEDLINE, CHEMICAL ABSTRACTS, KEYWORDS: CRYPTOSPORIDI? and ANTIBOD? and OOCYST?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
✓ X	WO 93/24649 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 9 December 1993. See for example page 7 lines 31-37 examples 14 & 15.	17, 19
Y		1, 2, 6-10
P X	JOURNAL OF EUKARYOTIC MICROBIOLOGY (1995 July - Aug) 42(4) 395-401 BONNIN A et al. "Monoclonal antibodies identify a subject of dense granules in Cryptosporidium parvum zoites and gamonts"	17, 19
P Y	See especially page 395 and page 400.	1, 2, 6-10

☐ Further documents are listed in the continuation of Box C ☐ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 December 1996	13.12.96
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (06) 285 3929	Authorized officer J.H. CHAN Telephone No.: (06) 283 2340

Form PCT/ISA/210 (second sheet) (July 1992) copjka

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.
PCT/AU96/00543

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
WO	93/24649	AU	44096/93	NZ	253620

END OF ANNEX